Printing Small Molecules as Microarrays and Detecting Protein−Ligand Interactions en Masse

Gavin MacBeath, Angela N. Koehler, and Stuart L. Schreiber*

Department of Chemistry & Chemical Biology
Center for Genomics Research and
Institute of Chemistry & Cell Biology
Howard Hughes Medical Institute, Harvard University
12 Oxford St., Cambridge, Massachusetts 02138

The ability to identify small molecule ligands for any protein of interest has far-reaching implications, both for the elucidation of protein function and for the development of novel pharmaceuticals. With the introduction of the split-pool strategy for synthesis[1] and the development of appropriate tagging technologies,[2] chemists are now able to prepare large collections of natural product-like compounds immobilized on polymeric synthesis beads.[3] These libraries provide a rich source of molecules for the discovery of new protein ligands.

With such libraries in hand, the availability of efficient methods for screening these compounds becomes imperative. One method that has been used is the on-bead binding assay.[4] An appropriately tagged protein of interest is mixed with the library and beads displaying cognate ligands are subsequently identified by a chromagenic or fluorescence-linked assay. Despite the utility of this approach, it is limited by the small number of proteins that can be screened efficiently. In principle, the beads can be stripped of one protein and re-probed with another; however, this serial process is slow and limited to only a few iterations. To identify a specific small molecule ligand for every protein in a cell, tissue, or organism, high-throughput assays that enable each compound to be screened against many different proteins in a parallel fashion are required.

To address this issue, we have developed a technique that we refer to as small molecule printing (SMP). First, synthesis beads are distributed into polypropylene microtiter plates at a density of one bead per well. The attached compounds are then released from their beads and dissolved in a small volume of a suitable solvent. Due to the minute quantities of compound present on each bead, extreme miniaturization of the subsequent assay is an absolute requirement. Taking our cue from cDNA microarray technology, we use a high-precision robot[5] to pick up a small volume of dissolved compound from each well and repetitively deliver approximately 1 nL of solution to defined locations on a series of chemically derivatized glass microscope slides (∼150 slides per print run). This results in the formation of microscopic spots of compound on the slides (200−250 μm in diameter). Each compound contains a common functional group that mediates covalent attachment to the slide surface. In this way, compounds are arrayed and subsequently immobilized on glass slides at extremely high spatial densities (>1000 spots per cm^2). Each slide can then be probed with a different tagged protein and binding events can be detected by a fluorescence-linked assay. While the presence of a linker connecting the small molecule to the slide reduces the number of binding modes available to each compound
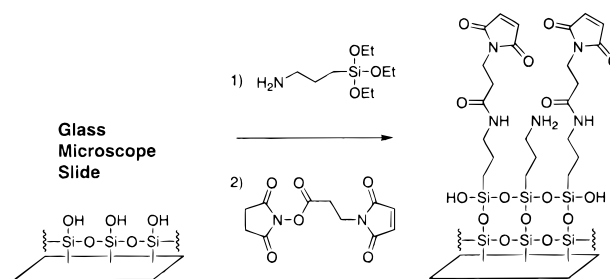

Figure 1. Preparation of maleimide-derivatized glass slides.

in the library, it also enables the selection of compounds with known sites of attachment. This information can facilitate the preparation of small-molecule dimerizers, the purification of target proteins by affinity chromatography, and the measurement of binding kinetics by surface plasmon resonance.

Successful implementation of this technique requires robust attachment chemistry. We have explored several approaches and currently favor the use of a Michael addition. Plain glass slides (2.5 cm × 7.5 cm) are derivatized as shown in Figure 1 to give surfaces that are densely functionalized with maleimide groups.[6] Thiol-containing compounds readily attach to the surface upon printing, presumably via the expected thioether linkage. A critical feature of the binding assay is the observed lack of nonspecific protein-binding to the slide surface in aqueous buffer, which we attribute to the hydrophilicity of the maleimide functionality.

To test the idea of SMP, we chose three unrelated molecules for which specific protein receptors are available. Compound **1** (R = OH) is the vitamin biotin, which is recognized by the bacterial protein streptavidin.[7] Compound **2** (R = OH) is a derivative of the steroid digoxigenin and is recognized by the mouse monoclonal antibody DI-22 (Sigma). Finally, compound **3** (R = OH) is a synthetic pipecolyl α-ketoamide, which was designed to be recognized by the human immunophilin FKBP12.[8] Each of these compounds was attached to 425 μm diameter polystyrene beads via a 6-aminocaproic acid linker and either 4-methoxytrityl-protected cysteine (X = S(Mmt)) or alanine (X = H; negative control). To create reference points on the slides, we also prepared beads with a thiol-labeled derivative of tetramethylrhodamine (**4a**). Individual beads were placed in 28 separate wells of a 96-well plate and the compounds were deprotected, cleaved, and subsequently dissolved in 5 μL of DMF. The released compounds were then arrayed robotically onto a series of maleimide-derivatized slides with a distance of 300 μm between the centers of adjacent spots. Each slide was printed according to the pattern illustrated in Figure 3D. After 12 h, the slides were blocked with 2-mercaptoethanol, washed extensively, and probed with different proteins.

The slide in Figure 3A was probed with Cy5-conjugated streptavidin, washed, and scanned using an ArrayWoRx fluorescence slide scanner. The slide was scanned for tetramethylrhodamine fluorescence (false-colored green) and Cy5 fluorescence (false-colored red). As anticipated, only the spots containing **1a** were visible when scanned for Cy5 fluorescence, indicating that localization of streptavidin on these spots was both specific for biotin and dependent on the thiol functionality. Using a two-step detection method, the slide in Figure 3B was probed first with DI-22 and then with a Cy5-conjugated goat-anti-mouse

(1) (a) Furka, A.; Sebestyén, F.; Asgedom, M.; Dibó, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 487−493. (b) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82−84.
(2) Nestler, H. P.; Bartlett, P. A.; Still, W. C. *J. Org. Chem.* **1994**, *59*, 4723−4724.
(3) Tan, D. S.; Foley, M. A.; Shair, M. D.; Schreiber, S. L. *J. Am. Chem. Soc.* **1998**, *120*, 8565−8566.
(4) Lam, K. S.; Lebl, M.; Krchnák, V. *Chem. Rev.* **1997**, *97*, 411−448.
(5) Schena, M.; Shalon, D.; Davis, R. W.; Brown, P. O. *Science* **1995**, *270*, 467−470.

(6) Experimental details are available via the Web at http://www-schreiber.chem.harvard.edu/home/protocols/SMP.html.
(7) Chaiet, I.; Wolf, F. J. *Arch. Biochem. Biophys.* **1964**, *106*, 1−5.
(8) Holt, D. A.; Luengo, J. I.; Yamashita, D. S.; Oh, H.-J.; Konialian, A. L.; Yen, H.-K.; Rozamus, L. W.; Brandt, M.; Bossard, M. J.; Levy, M. A.; Eggleston, D. S.; Liang, J.; Schultz, L. W.; Stout, T. J.; Clardy, J. *J. Am. Chem. Soc.* **1993**, *115*, 9925−9938.
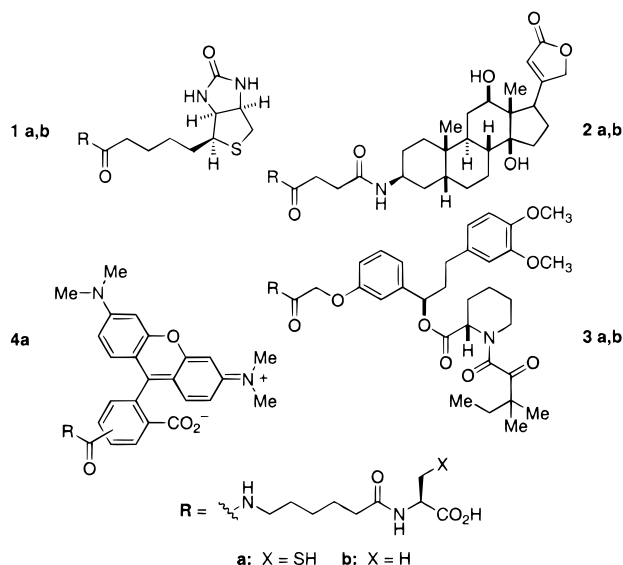
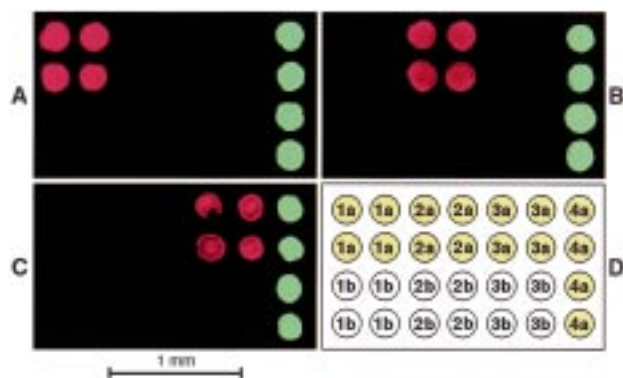**Figure 2.** Compounds used for small molecule printing.

Figure 3. Small molecules printed on maleimide-derivatized glass slides and detected with fluorophore-conjugated proteins.

antibody. As anticipated, the Cy5 fluorescence localized to the **2a**-containing spots. Finally, the slide in Figure 3C was probed using a three-step method: $(His)_6$-FKBP12 followed by mouse-anti-$(His)_6$ antibody followed by Cy5-conjugated goat-anti-mouse antibody. As before, the fluorescence localized to the appropriate spots. Of the three protein−ligand pairs used in these studies, the lowest affinity interaction was that observed between FKBP12 and compound **3** (estimated K_d of 8.8 nM). To explore further the limits of this assay, we also printed two lower-affinity ligands of FKBP12 (estimated K_d values of 140 nM and 2.6 μM). Like compound **3**, both ligands were detected specifically by FKBP12. Moreover, the intensity of the fluorescent spots correlated well with the affinity of the protein for the immobilized compounds (data not shown).

These results illustrate both the high selectivity and remarkable sensitivity of this slide-based assay. To illustrate the highly parallel nature of SMP, we released compound **1a** from a single 425 μm diameter polystyrene bead and dissolved the released compound in 10 μL of DMF. We repeated this procedure for compounds **2a** and **3a**. Using our microarraying robot, we repetitively spotted these three compounds in an alternating pattern on a single maleimide-derivatized slide, using the same spatial density as in Figure 3. Each compound was spotted 3600 times, using less than
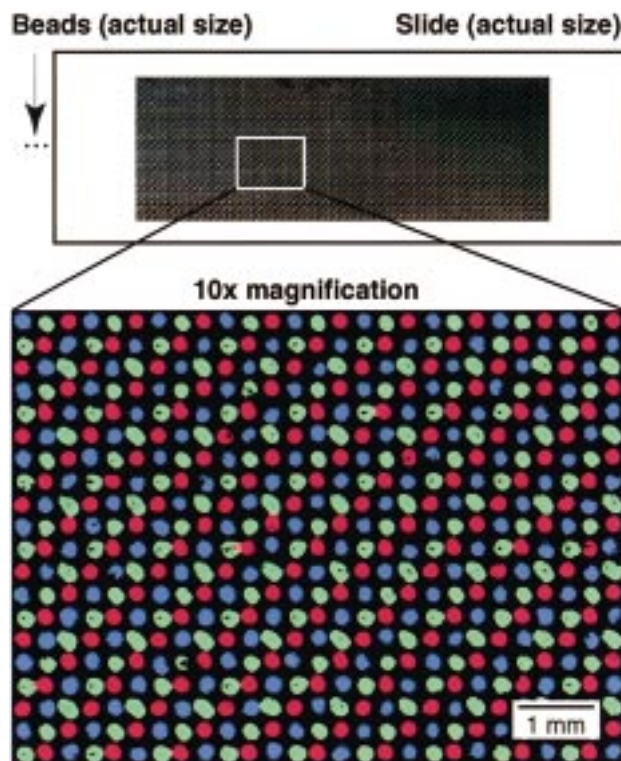

Figure 4. 10800 distinct spots on a single slide using compound released from only three beads.

half of the compound from each bead and yielding 10800 distinct spots. The slide was then probed in a single step with a solution containing FITC-conjugated streptavidin, Cy3-conjugated DI-22, and Cy5-conjugated FKBP12. Following a brief washing step, the slide was scanned for fluorescence (FITC, Cy3, and Cy5 were false-colored blue, green, and red, respectively). As shown in Figure 4, the three differentially labeled proteins localized to the spots containing their cognate ligands.

These results show that SMP allows thousands of binding assays to be performed in a parallel fashion using compound released from a single synthesis bead. This means that, in principle, thousands of identical microarrays can be constructed from a single split-pool library. With the development of techniques that enable the construction of libraries of expressed proteins from any organism or tissue of interest, we will soon have access to large arrays of recombinant proteins. Coupling these resources with small-molecule microarrays should aid in the eventual discovery of a small-molecule partner for every gene product.